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Allelic loss at the short arm of chromosome 11 is one of the most common and potent events in the progression and metastasis of breast cancer. Here, we present evidence that the Integrin-Linked Kinase (*ILK*) gene maps to the commonly deleted chromosome 11p15.5 and suppresses malignant growth of human breast cancer cells *in vitro*. *ILK* is expressed in normal breast tissue but not in metastatic breast cancer cell lines or in advanced breast cancers. Transfection of wild-type *ILK* into the MDA-MB-435 mammary carcinoma cells potently suppressed their growth and invasiveness *in vitro*. Conversely, expression of the ankyrin repeat or catalytic domain mutants of *ILK* failed to suppress the growth of these cells. Growth suppression by *ILK* is not due to apoptosis but is mediated by its ability to block cell cycle progression in the G1 phase. These findings directly demonstrate that *ILK* deficiency facilitates neoplastic growth and suggest a novel role for the *ILK* gene in tumor suppression.

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Pratima Karnik
PI - Signature

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A. INTRODUCTION:

Genetic alterations that occur in breast cancer are believed to be of importance for initiation as well as progression of the disease. These genetic alterations lead to the loss or activation of a number of critical genes, such as those involved in cell proliferation, differentiation, apoptosis, and genetic stability. The genetic abnormalities most frequently observed in breast tumors are amplification of proto-oncogenes (*MYC*, *ERBB2* and *CCND1*), mutations of *TP53*, and loss of heterozygosity (LOH) on chromosomes 3p, 6q, 7q, 8p, 9p, 11, 13q, 17, 18q and 22q (Driouch et al., 1998; Bieche et al., 1995). Metastatic phenotypes have been linked to such genes as *NME1* (17q), *CDH1* (16q), *BRMS1* (11q), and *KISS1* (1q) (Driouch et al., 1998; Siitonen et al., 1996; Seraj et al., 2000; Lee et al., 1996). LOH analyses have defined regions of deletion associated with metastasis on chromosomes 3p21, 15q14, 16q22 and 11p15 (Bieche et al., 1995; Karnik et al., 1998)

Frequent genetic alterations on chromosome 11p15 suggest a crucial role for this region in breast (Karnik et al., 1995; Karnik et al., 1998) and other adult (Fearon et al., 1985; Viel et al., 1992; Bepler et al., 1994; Lothe et al., 1989; Wang et al., 1988) and childhood cancers (Karnik et al., 1998; Besnard-Guerin et al., 1996; Henry et al., 1989; Koufos et al., 1985; Sotel-Avila et al., 1976). More recently, we have mapped two distinct regions on chromosome 11p15.5 that are subject to LOH during breast tumor progression and metastasis (Karnik et al., 1998). LOH at region 1 correlated with tumors that contain ductal carcinoma *in situ* suggesting that the loss of a critical gene in this region may be responsible for early events in malignancy. LOH at region 2 correlated with a more aggressive tumor and an ominous outlook for the patient, such as aneuploidy, high S-phase fraction and the presence of metastasis in regional lymph nodes. Although considerable advances have been made in the fine-mapping of chromosome 11p15.5, the tumor suppressor gene(s) encoded by this region have evaded identification.

Integrin-linked kinase (*ILK*) is an intriguing serine/threonine kinase that has been implicated in integrin-, growth-factor- and Wnt-signaling pathways (Dedhar et al., 1999). It binds to the cytoplasmic domains of β1 and β3 integrins and mediates the down-stream signaling events in integrin function (Hannigan et al., 1996). Interactions between integrins and their ligands are involved in the regulation of many cellular functions, including embryonic development, cell proliferation, tumor growth and the ability to metastasize (Hynes, 1992). In *Drosophila*, the absence of *ILK* function causes defects similar to loss of integrin adhesion and *ILK* mutations cause embryonic lethality and defects in muscle attachment (Zervas et al., 2001). Although *ILK* maps to the commonly deleted chromosome 11p, the potential of this gene to serve as a tumor suppressor has not been established. We have therefore analyzed the effect of *ILK* expression on the *in vitro* and *in vivo* tumor growth and invasion of human mammary carcinoma cells.

B. BODY:

Localization of *ILK* to the LOH region on chromosome 11p15.5

The LOH region 2 (Karnik et al., 1998) extends between the markers D11S1760-D11S1331 on chromosomal band 11p15.5 (Figure-1). We constructed a 500 kb genomic contig (Karnik et al, unpublished results) that includes the critical region between D11S1760 and D11S1331. Using a PCR-based screening method, we initially isolated PAC and BAC clones that contained D11S1760 and D11S1331 markers. The order of the genomic clones in the contig was confirmed by mapping of STSs, ESTs, unigene clusters and known genes that were previously mapped to chromosome 11. Eleven novel transcripts and seven previously reported genes were PCR-mapped to the critical region between D11S1760 and D11S1331. Three of the known genes, Tata box-binding protein-associated protein (*TAF II 30*) (Scheer et al., 1995), Lysosomal pepstatin insensitive protease (*CLN2*) (Sleat et al., 1997) and Integrin -linked kinase (*ILK*) (Hannigan et al., 1997) were previously mapped only at the level of cytogenetic resolution. However, with the current mapping data, we have been able to determine the precise genomic locations of

these three genes (Figure-1). The map location and its role in multiple signaling pathways makes ILK an attractive candidate tumor suppressor gene.

Loss of *ILK* expression in human breast carcinomas

To determine whether *ILK* has a role in breast cancer progression, mRNA expression in normal and tumor breast epithelial cells was compared by Northern blot hybridization (Figure-2). A single 1.8 kb *ILK* mRNA is highly expressed in all samples of normal breast epithelial cells. Three representative examples are shown in Figure-2 (N1, N7 and N8) In sharp contrast, there is complete loss of *ILK* mRNA expression in 9 out of 15 (~60%) invasive breast tumors and a 2-5 fold down-regulation of *ILK* mRNA in the remaining breast tumors (Figure-2A). Comparison of *ILK* mRNA expression in a panel of well-characterized breast cancer cell lines and in the non-malignant breast epithelial cell line MCF-10A is shown in Figure-2B. *ILK* mRNA expression in MCF-10A is comparable to the expression in normal breast tissue (N7, N8) (Figure-2B). However, there is a 3-5 fold down-regulation of *ILK* mRNA expression in the breast cancer cell lines MCF-7, T47D, ZR75.1, MDA-468, MDA-134, MDA-231 and MDA-435 (Figure-2B).

To further confirm these observations, *ILK* protein expression was also examined using indirect immunofluorescence microscopy in frozen samples of 20 normal and corresponding pathological human breast tissue samples. Figure-3 shows four representative examples. Immunohistochemical staining of normal breast tissue with *ILK*-specific primary antibody and rhodamine labeled secondary antibody shows specific staining of the mammary epithelial cells surrounding the lumen in normal breast tissue from breast cancer patients. *ILK* expression is particularly intense in epithelial cells both within large ducts and within terminal duct lobular units but not in the stromal compartment. Incubation with purified nonspecific rabbit immunoglobulin IgG, did not result in any positive staining of the normal epithelium of the breast (control). The normal breast tissue from four representative patients were positive, (3N, 12N, 6N and 10N) whereas ILK expression was nearly completely lost in the four corresponding infiltrating ductal carcinomas (3T, 12T, 6T, 10T) (Figure-3). These data show that *ILK* production by breast tumor cells correlates inversely with tumorigenecity and metastatic potential.

The *ILK* gene maps to chromosome 11p15.5 a region that displays a high frequency (~60%) of LOH in breast cancer. All breast tumor samples described in Figures 2 and 3 have previously been identified to contain LOH at the 11p15.5. (Karnik et al., 1998). Allelic loss results in the reduction of gene dosage and thus may result in decreased expression. However, as seen in Figure-2, all tumors have LOH for 11p15.5 and yet, only some tumors show complete loss of ILK expression. Therefore, intragenic mutations or epigentic mechanisms might contribute to the biallelic silencing of the ILK gene in breast tumors. We sought to determine if mutations are involved in the dysregulation of the *ILK* gene during the progression of human breast cancer. The *ILK* gene consists of 13 exons (Melchior et al., 2000, GenBank database, GI accession AJ404847). Primers derived from the sequences flanking each exon of *ILK* were used to analyze genomic DNA from 20 invasive breast tumors and matched normal tissue from the same patients. Using PCR-single strand conformation polymorphism (PCR-SSCP), only one of the 20 tumors analyzed showed a band shift in the SSCP assay. Subsequent DNA sequencing confirmed a silent mutation at codon 352 (GCA--->GCG) (data not shown). These results demonstrate that *ILK* mRNA and protein expression is consistently down-regulated during the progression of human breast cancer and this down-regulation does not commonly involve mutations. Epigenetic mechanisms as a probable cause of *ILK* gene silencing are currently under investigation.

***ILK* suppresses cell growth in human breast carcinoma cells**

The inverse correlation between *ILK* expression and tumorigenecity suggested the hypothesis that elaboration of *ILK* by tumor cells into their environment may exert an inhibitory effect. To test this hypothesis, we transfected the human breast carcinoma cell

line MDA-MB-435 with the *ILK* cDNA. This cell line synthesizes very low levels of *ILK* compared to normal mammary epithelial cells (Figure-2B) and can be injected into the mammary fat pad of nude mice to provide an orthotopic model system for human breast cancer tumorigenecity and metastasis. The MDA-MB-435 cells were transfected with a mammalian expression vector pIRES-EGFP containing full length *ILK* cDNA under control of the CMV promoter. A total of four stable clones expressing different levels of *ILK* have been established. Comparison of mRNA expression by Northern blot analyses revealed that the clones TR4 and TR5 expressed slightly higher levels of *ILK* mRNA compared to the clones TR2 and TR3 (Figure 4B). Based on Northern analysis, *ILK* expression in clone TR5 is 2-3 fold higher compared to the expression in the non-malignant breast epithelial cell line MCF-10A and to the expression in normal mammary epithelial cells (Figure-2B) suggesting that ILK is overexpressed in the TR5 clone. The expression of *ILK* in empty vector controls (data not shown) is comparable to untranfected MDA-MB-435 cells (UT). *ILK* protein levels in transfected (TR5) and untransfected cells was determined by indirect immunofluorescence. High levels of *ILK* protein are expressed in the transfected MDA-MB-435 cells (Figure 4Ac) compared to the untransfected control (Figure-4Ab). The *ILK* protein is localized in the cytoplasm. Most strikingly, corresponding to the low levels of *ILK* mRNA (Fig. 2B), the highly metastatic MDA-MB-435 cell line showed very little detectable *ILK* protein (Figure 4Ab).

To determine whether *ILK* overexpression had any effect on the growth properties of the MDA-MB-435 cells, we determined the growth kinetics of the clones TR3 and TR5. *ILK* expression causes the MDA-MB-435 cells to grow to a low saturation density (Figure-5A) and there is substantial growth suppression of the TR5 clone compared to untransfected MDA-MB-435 cells. The growth suppression of the transfectants was *ILK* concentration dependent with TR5 (high expressing clone) growing to a lower saturation density than TR3 (low expressing clone). Furthermore, the growth rate of TR5 was decreased by ~ 40% with a cell doubling time of 96 hours compared to the growth rate of cells transfected with vector alone or untransfected cells which had a doubling time of 48 hours.

The ability of *ILK* to suppress growth could be due to a non-specific lethal effect of protein overproduction. Alternatively, it could be a manifestation of a more specific effect on cell proliferation. To further investigate these possibilities and to establish a link between a functional *ILK* and growth suppression, we tested the growth kinetics of two *ILK* variants. *ILK* contains four ankyrin repeats at the NH2-terminus (Dedhar et al., 1999) that participate in protein-protein interactions important for integrin-, growth-factor- and Wnt- mediated signaling. First, a deletion mutant, Δ ANK lacking this domain was constructed. In addition, the residue E359 has been shown to be essential for *ILK* function (Dedhar et al., 1999). We therefore constructed an *ILK* point mutant (E359K) in which the highly conserved Glu359 within the *ILK* catalytic domain was substituted with lysine. The growth rates of the stably transfected *ILK* mutant clones Δ ANK and E359K compared to the *ILK* transfectant TR5 are shown in Figure 5B. As discussed above, overexpression of the wild-type *ILK* strongly inhibited growth of the MDA-MB-435 cells. In contrast, both the Δ ANK and E359K mutants lost their capacity to suppress the growth of the MDA-MB-435 cells (Figure-5B) arguing against a non-specific effect of protein overproduction.

Expression of *ILK* in MDA-MB-435 Cells Leads to a G1 Cell Cycle Arrest

The observed growth suppression by *ILK* could be caused by either increased apoptosis or inhibition of cell proliferation. To investigate the mechanisms underlying the growth suppression by *ILK* expression, we studied apoptosis by fluorescence-activated cell sorting (FACS) analysis of Annexin-V stained *ILK* and vector transfectants. There was no increase in the rate of apoptosis in *ILK*-expressing cells compared to vector transfectants (data not shown). Therefore, programmed cell death does not seem to account for the growth suppression of *ILK* transfected cells.

To test for cell cycle regulation by ILK, propidium iodide stained MDA-MB-435 clones were analyzed by flow cytometry. Expression of ILK increased the number of cells in G0/G1 from 64 to 85% (Figure-5C, VT and TR5-ILK) and decreased inversely the number of cells in S and G2/M phase from 26 and 10% to 9 and 5% (Figure-5C, VT and TR5-ILK). In contrast, the cell cycle profiles of the two ILK variants Δ ANK and E359K were very similar to the parental MDA-MB-435 cells. These results indicate that ILK growth suppression results from G1 cell cycle arrest. The accumulation of cells in the G0/G1 phase of the cell cycle suggests arrest predominantly at the G1/S boundary. ILK overexpression does not induce cell death or apoptosis but induces a very pronounced growth arrest with 85% of the cells in G0/G1, a property that is the hallmark of growth/tumor suppressors. Growth suppressor genes play an important role in checkpoint function and loss of genes associated with checkpoint functions seem to have important implications in the development of cancer. The percentage G1 arrest induced by *ILK* is comparable to the effect on cell cycle progression induced by the *p21* cyclin-dependent kinase inhibitor (Yang et al., 1995).

ILK Suppresses the Invasive Phenotype of Human Breast Carcinoma Cells

The invasiveness of tumor cells represents one of several important properties necessary for the formation of metastases. Cell migration on vitronectin in vitro has been linked to the metastatic capacity of tumor cells in vivo (Nip et al., 1992; Brooks et al., 1997). To examine the effects of *ILK* expression on breast cancer cell invasion, the ability of vector and *ILK* transfected MDA-MB-435 cells to degrade and invade vitronectin -coated polycarbonate membrane was investigated. As shown in Figure-6A, a significant reduction in invasive potential was noted in the ILK expressing clone TR5 (ILK) compared to vector transfected MDA-MB-435 cells (VT) (Figure-6A). Cell invasion through membranes coated with vitronectin, is decreased by 60% in MDA-MB-435 cells expressing *ILK* compared to vector transfected MDA-MB-435 cells. In contrast, the two ILK variants Δ ANK and E359K have no significant effect on cell invasion under identical conditions (Figure-6A). In fact, there is a slight increase in invasive potential of the variant clones (Δ ANK and E359K), suggesting a dominant-negative effect, perhaps due to inhibition of endogenous ILK in the MDA-MB-435 cells. These results indicate that *ILK* expression abates extracellular matrix invasion of tumor cells *in vitro*, one of the hallmarks of tumorigenecity and transformed cell growth.

Cell adhesion, migration and invasion are controlled by the levels of integrins and by the amount of fibronectin matrix around the cell (Hynes, 1992). Because the αvβ3 and α5ß1 integrins have been implicated in the regulation of angiogenesis, tumor cell migration, invasion and metastasis, we speculated that *ILK* might regulate cell migration via alteration of the cellular composition of integrins. Using a panel of specific antibodies against these integrins in flow cytometry analysis, we compared integrin expression patterns in relation to the *ILK* expression status. The results are shown in Figure-6B. The *ILK* transfected cells demonstrated a 22% increase in levels of the growth-suppressing integrin α5ß1 and a 31% decrease in levels of the growth-promoting integrin αvβ3 compared to the control cells. The changes in levels of αvβ3 and α5ß1 expression in *ILK* transfected cells although relatively moderate in comparison to control cells, nonetheless, were highly significant. Collectively, these observations suggest that *ILK* reduces the invasive potential of MDA-MB-435 cells by altering their integrin profiles, which changes their ability to perceive and interact with their extracellular environment.

C. CONCLUSIONS:

Growth inhibitory functions of *ILK*

The present study reveals that expression of *ILK* potently suppresses the growth and tumorigenecity of the human mammary carcinoma cells MDA-MB-435 *in vitro*. This growth suppression activity requires a functional *ILK* protein, since expression of wild-type *ILK*, but not the ankyrin repeat or the catalytic domain mutants, resulted in growth

suppression of MDA-MB-435 cells. The demonstration of a growth suppressive function establishes *ILK* as a tumor suppressor gene and directly implicates its loss in processes regulating the growth and maintenance of the malignant phenotype in human breast cancer. Our results strongly suggest that the growth suppression by *ILK* is not due to apoptosis but is mediated by its ability to block cell cycle progression at G1 phase. During this process, the neoplastic cells cease to proliferate and lose their ability to migrate through vitronectin membranes.

ILK seems to play a dual role in the MDA-MB-435 model system. First, it regulates cell-cycle progression at the G1/S boundary and second, it modulates the levels of integrins, transmembrane receptors that have been shown to regulate cell growth, survival, and differentiation. Like many tumor suppressor genes such as *p53, APC, p16INK4a* and *p21, ILK* arrests tumor cell growth by blocking cell-cycle progression in the G0/G1 phase. Growth suppressor genes play an important role in checkpoint function and silencing of genes associated with checkpoint functions seem to have important implications in the development of cancer. Integrin signals are necessary for cells to traverse the cell division cycle. Progression through the G1 phase of the cell cycle requires the sequential activation of the cyclin-dependent kinases *(Cdk's) Cdk 4/6 and Cdk 2* and the activities of these kinases are regulated by integrins (Giancotti and Ruoslahti., 1999). In view of our observation that *ILK* regulates cell-cycle progression at the G1 phase, it is quite probable that the integrin interactions with the *Cdk's* are mediated by *ILK*. *ILK* could interact with specific integrin cytoplasmic domains and couple them to appropriate downstream signaling pathways. This in turn could regulate such functions as coordination of growth factor signals and altering gene expression required for cell proliferation and differentiation.

The interaction of cells with the surrounding extracellular matrix (ECM) affects many aspects of cell behavior, including the migratory properties of cells, their growth, and differentiation (Giancotti and Ruoslahti., 1999). Integrins are transmembrane heterodimeric proteins that mediate such interactions. The large extracellular part of both α and ß subunits bind proteins within the ECM. The short cytoplasmic domain of the β integrin subunit anchors the cytoskeleton to the plasma membrane via intermediary adaptor proteins. In *Drosophila* (Zervas et al., 2001), *ILK* has been shown to be a component of the structure linking the cytoskeleton and plasma membrane at sites of integrin-mediated adhesion. The absence of *ILK* function in *Drosophila* causes defects similar to loss of integrin adhesion. Similarly, the downregulation of *ILK* expression in mammary epithelial cells could cause the cells to become more invasive. Indeed, as seen in our present study, *ILK* overexpression in the highly metastatic breast cancer cell line MDA-MB-435 causes the cells to lose their tumorigenecity and metastatic potential. What is the biological significance of *ILK*-mediated regulation of the α5ß1 and αvβ3 integrins? Previous studies have shown that α5ß1 expression is frequently lost during malignant progression, a phenomenon that has been observed in human colonic, mammary and pancreatic cancer (Ruoslahti, 1999, Ruoslahti, 1996). Expression of the α5ß1 integrin in HT29 human colon carcinoma cells also blocks tumorigenecity in nude mice (Varner and Cheresh, 1996). In contrast, the αvβ3 integrin cooperates with certain growth factors, potentiating their effects on cells and its expression correlates with a role in metastasis. Indeed, expression of integrin αvβ3 is significantly higher in breast tumors of patients with metastases than in those without metastasis and may have a role in skeletal metastases (Liapis et al., 1996). Therefore, our observation that *ILK* modulates the levels of α5ß1 and αvβ3 integrins is very significant and suggests that *ILK* may reduce the invasive potential of MDA-MB-435 cells by altering their integrin profiles.

Frequent Down-Regulation and Lack of Mutations of the ILK Gene in Breast Carcinoma

We determined by Northern blot and immunohistochemical analysis that most invasive breast carcinomas exhibit complete loss or very low expression of *ILK* mRNA and protein. However, in our present study, we detected no homozygous deletions or intragenic mutations in the *ILK* gene. Thus, it is likely that the *ILK* gene is not a target for mutations in many cancers, and other mechanisms for *ILK* down-regulation should be considered. *ILK* maps to chromosome 11p15.5, a region that exhibits a high frequency (40-60%) of LOH in breast and other adult and childhood tumors (Karnik et al., 1998). It is thought that LOH alone cannot completely suppress *ILK* expression, as many genes can be expressed monoallelically (Bix et al., 1998 Hollander et al, 1998). Although all breast tumors used in this study were previously described (Karnik et al., 1998) to have LOH at 11p15.5, a small number of breast tumors still express *ILK* suggesting that *ILK* can be expressed monoallelically. Biallelic inactivation of the *ILK* gene could result either from epigenetic inactivation of both parental alleles or from epigenetic modification of one allele and loss of the second allele via mechanisms that result in LOH. Indeed, the *p16/CDKN2* and the *p15INK4B* cell cycle regulator genes are located at a region of high LOH on chromosome 9p21 and individual alleles in neoplasia are selectively silenced by promoter hypermethylation (Herman et al., 1995; Gonzalez-Zaluets et al., 1995). While the *Maspin* tumor suppressor gene is biallelically inactivated by aberrant cytosine methylation and heterochromatinization of the promoter (Domann et al., 2000)), the down-regulation of the *KAI1* metastasis gene involves neither mutations nor promoter hypermethylation. (Dong et al., 1996; Jackson et al., 2000). It has been suggested that there is a group of tumor suppressor genes that are unrecognized because the primary mechanism for their silencing is not known. Such genes may affect the cancer cell phenotype by expression changes and have been classified as Class II tumor suppressor genes (Sager., 1997). The molecular basis for the down-regulation of the *ILK* tumor suppressor gene in breast cancer is currently under investigation. The loss of expression that occurs during malignant progression of primary breast tumors suggests that *ILK* has potential value as a prognostic marker. Future studies should test the prognostic value of *ILK* on a larger scale, in order to establish more firmly a correlation between loss of *ILK* expression and progression of breast and other cancers.

The paradoxical effect of *ILK* on tumorigenecity.

Previous studies have shown that *ILK* overexpression results in loss of cell-cell adhesion (Novak et al., 1998), promotes suppression of anoikis by activation of *PKB/Akt* signaling (Attwell et al., 2000) and oncogenic transformation of the rat intestinal epithelial cells by activation of the *LEF-1/ß-catenin* signaling pathways (Radeva et al., 1997; Wu et al., 1998). In contrast, recent studies in *Drosophila* (Zervas et al., 2001) have shown that *ILK* is required for integrin-mediated adhesion, but not for signaling involving *ß-catenin (armadillo)* or *PKB*. *ILK* mutations in *Drosophila* cause embryonic lethality and defects in muscle attachment, and clones of cells lacking ILK in the adult wing fail to adhere, forming wing blisters. The *ILK* coding sequence is highly conserved in different species (Zervas et al., 2001), suggesting that it has an essential biological function in evolution. Our present data is consistent with the observations made in *Drosophila*. We have shown that transfection of the MDA-MB-435 mammary carcinoma cells with the *ILK* gene reduced the cells' ability to induce tumors and to invade through vitronectin membranes *in vitro*. The down-regulation of *ILK* in metastatic breast cancer cell lines and invasive breast tumors strongly suggests that *ILK* might block uncontrolled cell growth in normal breast tissue and that its absence may be permissive for malignant tumor growth. The negative correlation between *ILK* expression and growth suppression is unexpected when considered with the current concept that kinases are positively associated with tumorigenesis (for example, c-erB2). However, Lynch et al.(1999) have demonstrated that *ILK* is not a typical protein kinase and lacks a DFG motif or a conserved substitute for the catalytic aspartate residue

found in other kinases and they and other investigators (Balendran et al., 1999) have failed to detect protein kinase activity in *ILK* immunoprecipitates. Recent evidence (Lynch et al., 1999) suggests that *ILK* does not possess serine-473 kinase activity but functions as an adaptor to recruit either a serine-473 kinase or phosphatase. Mutations in the kinase domain shown to inactivate the kinase activity of human *ILK* do not show any phenotype in *Drosophila* (Zervas et al., 2001), suggesting a kinase independent function for *ILK*. Thus, it is likely that the functions of ILK are more complex than previously envisioned; and the divergent and often paradoxical effects mediated by *ILK* may depend on the particular cell-type, the cell-specific integrins that are activated by a cell, and on whether the adaptor protein *ILK* activates a serine-473 kinase or phosphatase.

In conclusion, we have shown that the loss of *ILK* expression is associated with the acquisition of a malignant breast tumor phenotype and that *ILK* may directly act as a tumor suppressor, presumably by controlling cell division. The absence of the *ILK* tumor suppressor protein, may promote uncoordinated G1 cell cycle progression, allowing cells to bypass the normal signaling processes regulated by growth factors and cell anchorage, leading to tumorigenesis. This novel information regarding the biological effects of *ILK* provides hopeful therapeutic utility for this potent tumor suppressor gene in the management of breast cancer.

D. KEY RESEARCH ACCOMPLISHMENTS:

- Chromosome 11 harbors a breast cancer tumor/metastasis suppressor gene
- Integrin linked kinase (ILK) is a key candidate gene that maps to this region
- ILK expression is downregulated in breast carcinomas that metastasize
- ILK expression inhibits the *in vitro* growth of the metastatic breast cancer cell line MDA-MB-435
- Growth suppression by *ILK* is not due to apoptosis but is mediated by its ability to block cell cycle progression in the G1 phase.
- ILK functions as a tumor suppressor gene in breast cancer (manuscript submitted for publication).

E. REPORTABLE OUTCOMES:

Allelic loss at the short arm of chromosome 11 is one of the most common and potent events in the progression and metastasis of breast cancer. We present evidence that the Integrin-Linked Kinase (*ILK*) gene maps to the commonly deleted chromosome 11p15.5 and suppresses malignant growth of human breast cancer cells both *in vitro*. *ILK* is expressed in normal breast tissue but not in metastatic breast cancer cell lines or in advanced breast cancers. Transfection of wild-type *ILK* into the MDA-MB-435 mammary carcinoma cells potently suppressed their growth and invasiveness *in vitro*, and reduced the cells' ability to induce tumors and metastasize in athymic mice. Conversely, expression of the ankyrin repeat or catalytic domain mutants of *ILK* failed to suppress the growth of these cells. Growth suppression by *ILK* is not due to apoptosis but is mediated by its ability to block cell cycle progression in the G1 phase. These findings directly demonstrate that *ILK* deficiency facilitates neoplastic growth and suggest a novel role for the *ILK* gene in tumor suppression.

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FIGURE LEGENDS:

Figure-1: Localization of *ILK* gene to the tumor suppressor region (LOH region 2) on chromosome 11p15.5. A transcript map of the LOH region is schematically represented with the relative location of the polymorphic markers, known genes, unigene clusters and expressed sequence tags (EST's).

Figure-2: Northern blot analysis of *ILK* mRNA expression. (A) Total RNA was isolated from normal breast tissue (N1, N7, N8), and fifteen invasive breast tumors (T1 to T15). B) Total RNA from exponentially growing non-malignant (MCF-10A), non-metastatic (MCF-7, T47D, ZR75.1, MDA-468 and MDA-134) and metastatic (MDA-435, MDA-231) breast cancer cell lines and normal breast tissue (N7, N8) was hybridized with ^{32}P-labeled *ILK* probe. Hybridization with the β-actin probe serves as control.

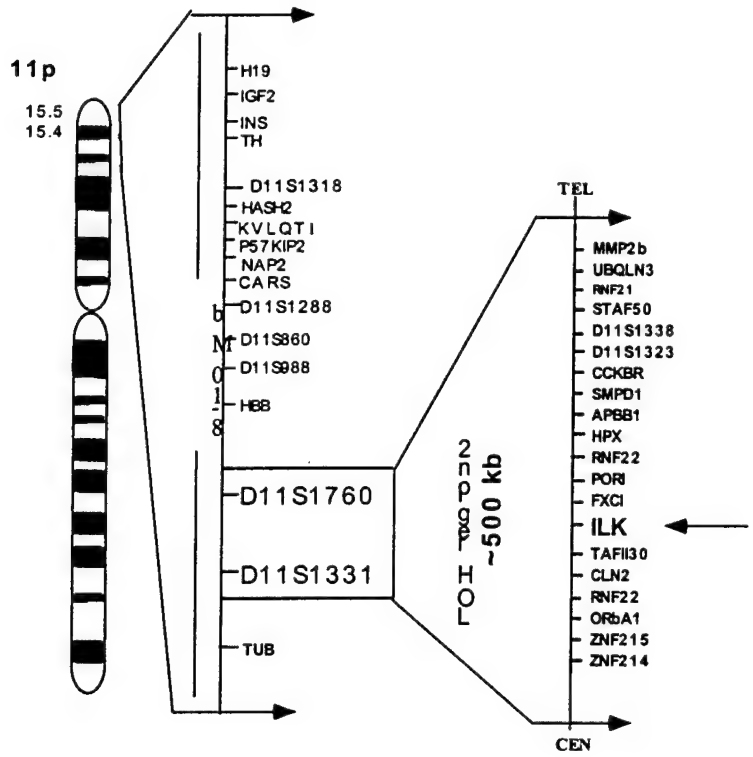
Figure-3: Immunohistochemical detection of *ILK* expression in normal breast tissues (3N, 12N, 6N, 10N) and corresponding invasive ductal carcinomas (3T, 12T, 6T, 10T). Normal ducts were positive for *ILK* expression whereas the invasive ductal carcinomas expressed little or no *ILK*. Control-Normal tissue minus primary antibody.

Figure-4: MDA-MB-435 cells were transfected with pIRES-EGFP vector containing full length *ILK* cDNA and four stable clones were isolated. (A) Immunohistochemical analysis of *ILK* expression in the MDA-MB-435 cells (b) before and (c) after transfection (stable clone TR5-*ILK*) (a) no primary antibody control. (B) Northern blot analysis of parental (UT) and *ILK* transfected MDA-MB-435 cells. TR2, 3, 4 and 5 represent stable *ILK* expressing clones. mRNA expression was determined by hybridization with ^{32}P-labeled *ILK* probe. β-actin expression serves as control.

Figure-5: Growth effects of wild type and mutant alleles of *ILK* in MDA-MB-435 breast cancer cells. The MDA-MB-435 cells were transfected with either full-length *ILK* cDNA, *ILK* mutant ΔANK, *ILK* mutant E359K or eukaryotic expression vector and stable clones were obtained. (A) Growth rates of two stable *ILK* expressing MDA-MB-435 cell clones (TR3-*ILK* and TR5-*ILK*) that contain full length *ILK* cDNA compared with a stable clone containing empty vector (VT) and untransfected MDA-MB-435 cells (UT). (B) Growth rates of *ILK* mutants ΔANK and E359K compared with the wild type *ILK* expressing clone TR5-*ILK*. The means of three independent experiments are shown. Bars represent SE. (C) Cell-cycle analysis by propidium iodide staining in MDA-MB-435 cells (UT), transfected with empty vector (VT), with full length *ILK* cDNA (TR5-*ILK*) or with the *ILK* mutants ΔANK and E359K. The regions between the vertical lines from left to right represent cells in G0/G1, S and G2/M respectively.

Figure-6: Cell invasion assay of MDA-MB-435 cells transfected with vector (VT), full length *ILK* and its variants (ΔANK, E359K). Cell invasion through vitronectin was analyzed using a modified Boyden chamber. Cells that invaded to the lower surface of the membrane were lyzed and absorbance determined at 560 nm. (B) Flow cytometric analysis of α5β1 and αvβ3 integrins expressed on the surface of *ILK* transfected and parental MDA-MB-435 cells. The relative fluorescence intensity of cells stained with α5β1 and αvβ3 antibodies is represented as percentage of cell shift. Bars represent S.E.

APPENDIX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15.4
H19
IGF2
INS
TH
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HASH2
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P57KIP2
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D11S860
D11S988
HBB
D11S1760
D11S1331
TUB
TEL
MMP2b
UBQLN3
RNF21
STAF50
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CCKBR
SMPD1
APBB1
HPX
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ZNF215
ZNF214
CEN
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Figure 1

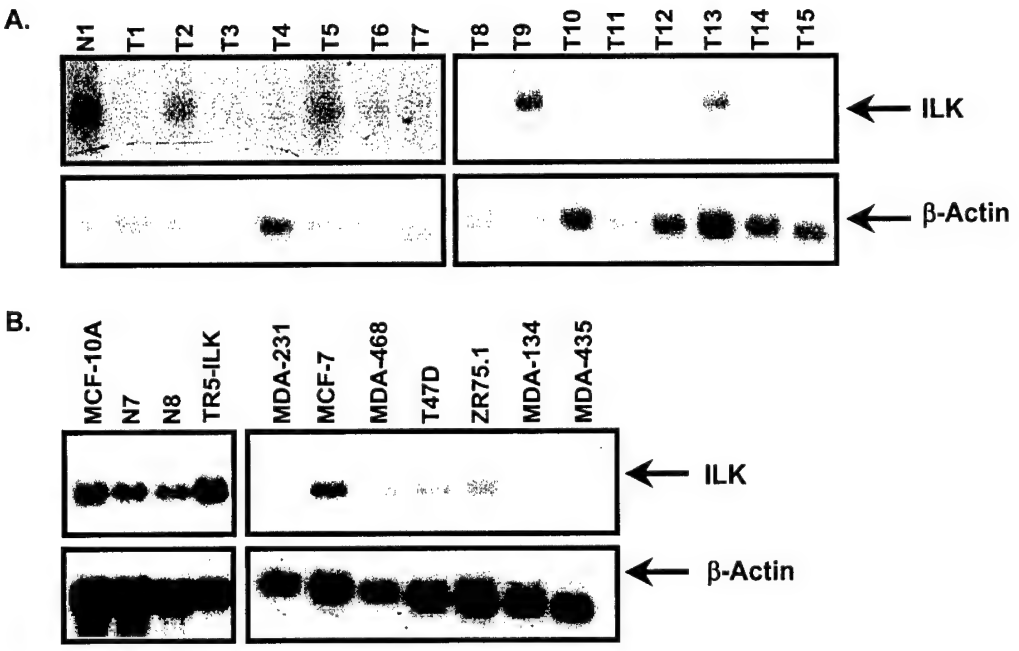

A.
N1
T1
T2
T3
T4
T5
T6
T7
T8
T9
T10
T11
T12
T13
T14
T15
ILK
β-Actin
B.
MCF-10A
N7
N8
TR5-ILK
MDA-231
MCF-7
MDA-468
T47D
ZR75.1
MDA-134
MDA-435
ILK
β-Actin

Figure 2

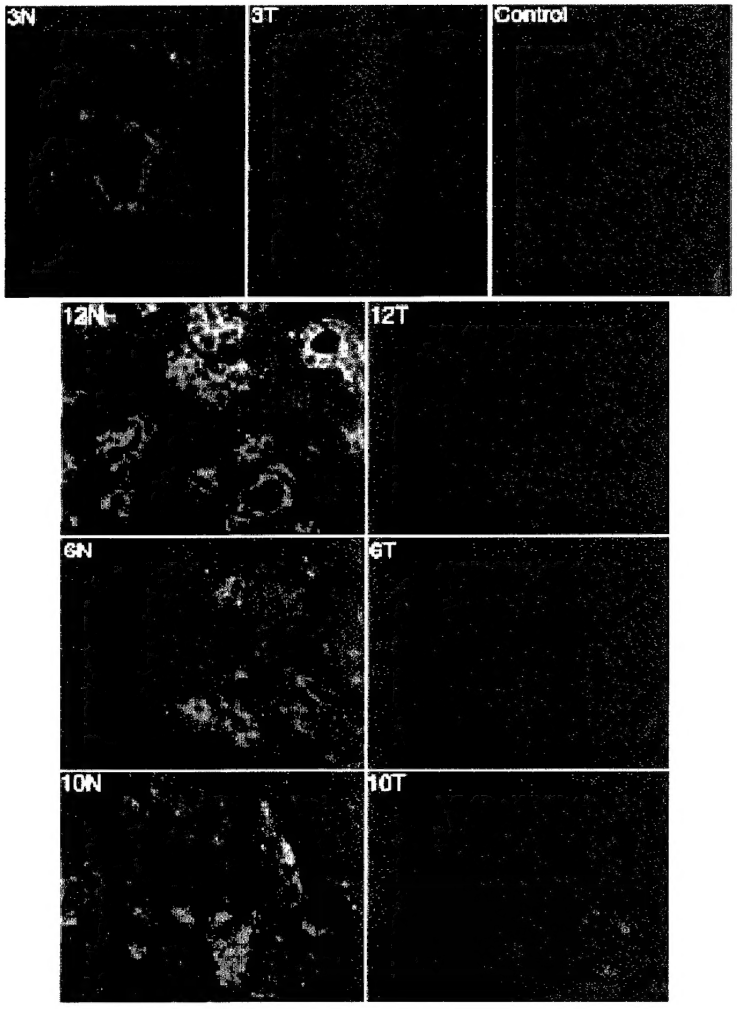
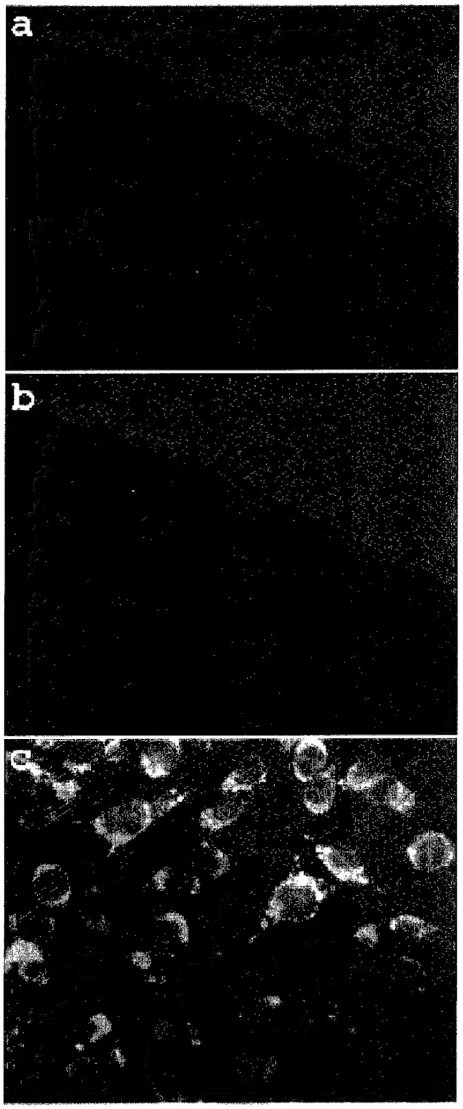


Fig. 3 Immunohistochemical detection of ILK expression in normal breast tissues (3N, 12N, 6N, 10N) and corresponding invasive ductal carcinomas (3T, 12T, 6T, 10T). Normal ducts were positive for ILK expression whereas the invasive ductal carcinomas expressed little or no ILK. Control- Normal tissue minus primary antibody.

A.



B.

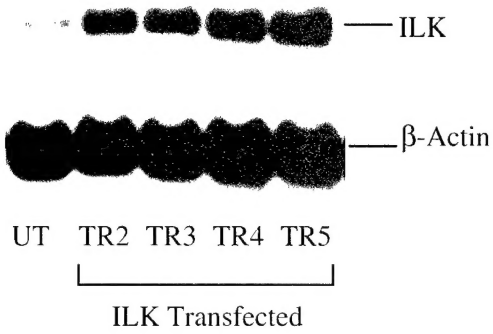


Fig. 4 MDA-MB-435 cells were transfected with pIRES2-EGFP vector containing full length ILK cDNA and four stable clones were isolated. (A) Immunohistochemical analysis of ILK expression in the MDA-MB -435 cells (b) before and (c) after transfection (stable clone TR5-ILK) (a) no primary antibody control. (B) Northern blot analysis of Parental (UT) and ILK transfected MDA-MB-435 cells. TR2,3,4 and 5 represent stable ILK expressing clones. mRNA expression was determined by hybridization with ^{32}p labeled ILK probe. β-Actin serves as control.

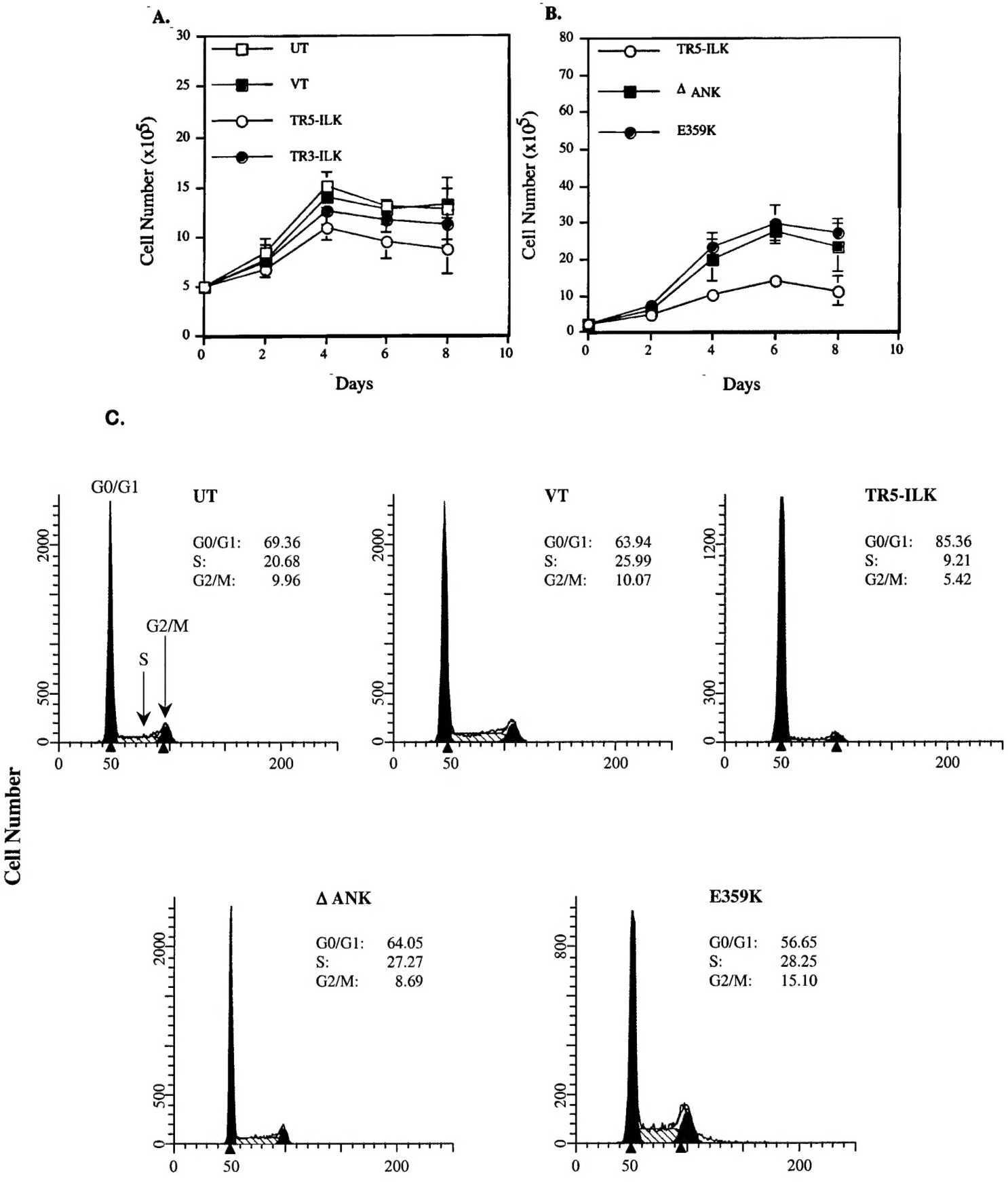

A.
UT
VT
TR5-ILK
TR3-ILK
Cell Number (x10^5)
Days
B.
TR5-ILK
Δ ANK
E359K
Cell Number (x10^5)
Days
C.
G0/G1
G2/M
S
UT
G0/G1: 69.36
S: 20.68
G2/M: 9.96
VT
G0/G1: 63.94
S: 25.99
G2/M: 10.07
TR5-ILK
G0/G1: 85.36
S: 9.21
G2/M: 5.42
Δ ANK
G0/G1: 64.05
S: 27.27
G2/M: 8.69
E359K
G0/G1: 56.65
S: 28.25
G2/M: 15.10
Cell Number
Fluorescence Intensity

Figure 5

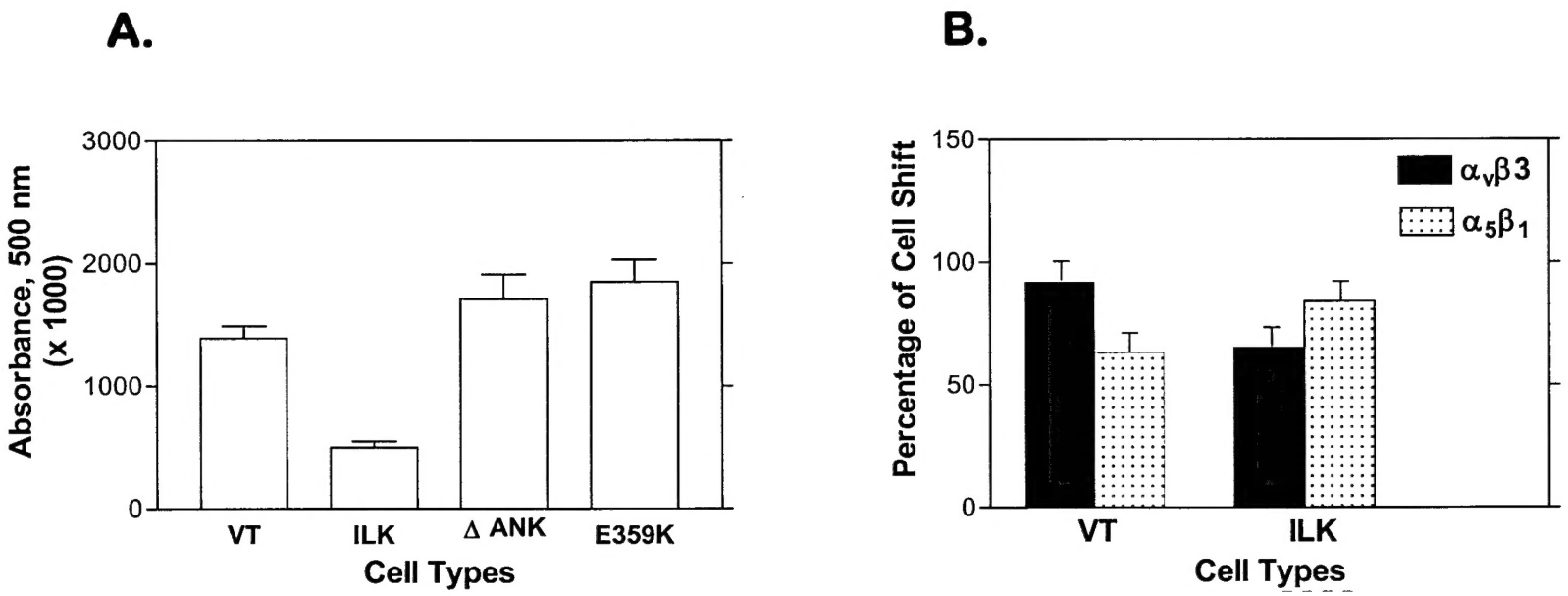

A.
Absorbance, 500 nm (x 1000)
3000
2000
1000
0
VT
ILK
Δ ANK
E359K
Cell Types
B.
Percentage of Cell Shift
150
100
50
0
αvβ3
α5β1
VT
ILK
Cell Types

Figure 6